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Phagocytic cells metabolize 25-hydroxyvitamin D_3 to 10-oxo-19-nor-25-hydroxyvitamin D_3 and a new metabolite, 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one

Takamune Hayashi, Sachiko Yamada*, Chisato Miyaura, Hirofumi Tanaka, Keiko Yamamoto*, Etsuko Abe, Hiroaki Takayama* and Tatsuo Suda

Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 and **Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan*

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The metabolism of 25-hydroxyvitamin D_3 [25(OH)D_3] was examined in several phagocytic cells including alveolar macrophages and myeloid leukemia cells (M1, HL-60 and U937). Phagocytic cells converted 25(OH)D_3 to 10-oxo-19-nor-25-hydroxyvitamin D_3 and a new metabolite. The former metabolite was dominant in shorter incubation periods (1 h), whereas the latter dominated over longer incubation periods (24 h). The new metabolite was produced from 25(OH)D_3 directly but not through 10-oxo-19-nor-25-hydroxyvitamin D_3. The new metabolite was unequivocally identified as 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one. These results suggest that phagocytic cells somehow promote oxidation of the triene part of vitamin D compounds.

Vitamin D metabolism; Phagocytic cell; Macrophage; 10-Oxo-19-nor-25-hydroxyvitamin D_3; 8α,25-Dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one

1. INTRODUCTION

Recently, much attention has been focused on the metabolism of vitamin D_3 in phagocytic cells. Phagocytic cells such as human blood leukocytes [1], monocytes [2] and tissue macrophages [1–3], and their transformed cells (HL-60 and U937) [4–7] have been reported to metabolize 25-hydroxyvitamin D_3 [25(OH)D_3] to more polar metabolites including 10-oxo-19-nor-25-hydroxyvitamin D_3 [10-oxo-19-nor-25(OH)D_3]. This metabolite has two isomers, 5*E* and 5*Z* forms. The former structure is similar to that of 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2D_3$], the active form of vitamin D_3, because the 3β-hydroxyl function of (5*E*)-10-oxo-19-nor-25(OH)D_3 occupies the position of the 1α-hydroxyl function of 1α,25(OH)$_2D_3$. These results led us to consider that the 5*E* form of 10-oxo-19-nor-25(OH)D_3 might be responsible for inducing monocytic differentiation. It has also been pointed out that this metabolite migrates at a similar position to 1α,25(OH)$_2D_3$ in a straight-phase high-pressure liquid chromatography (HPLC) system using 10% 2-propanol in hexane, the traditional chromatographic system for separating 1α,25(OH)$_2D_3$. However, neither the role nor the regulation of its production has been established.

In the course of investigating the metabolism of 25(OH)D_3 in phagocytic cells, we found a new metabolite of 25(OH)D_3 which migrated at a similar position to 24*R*,25-dihydroxyvitamin D_3 [24*R*,25(OH)$_2D_3$] in four different HPLC systems. The metabolite has now been identified as 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one. Here, we examined the relationship be-

Correspondence address: T. Suda, Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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tween the production of the two metabolites, 10-oxo-19-nor-25(OH)D_3 and the new metabolite, in phagocytic cells.

2. MATERIALS AND METHODS

2.1. *Animals and drugs*

Male mice, 6–8-week-old ddy strain, were obtained from Shizuoka Laboratory Animal Center (Shizuoka). 25(OH)D_3, 1α,25(OH)$_2D_3$ and 24*R*,25(OH)$_2D_3$ were kindly donated by Dr I. Matsunaga (Chugai Pharmaceutical Co., Tokyo). (5*E*)- and (5*Z*)-10-oxo-19-nor-25(OH)D_3 and 25,26*S*-dihydroxyvitamin D_3 [25,26*S*(OH)$_2D_3$] were synthesized in our laboratory. 25(OH)-[26,27-^{3}H]D_3 (spec. act. 20.6 Ci/mmol) was obtained from Amersham (England).

2.2. *Cells*

Alveolar macrophages were collected by the tracheobronchial lavage method and purified as reported in [8]. The murine myeloid leukemia cell line (M1, clone T22) was kindly donated by Dr M. Hozumi (Saitama Cancer Center Research Institute, Saitama). The human promyelocytic leukemia cell line (HL-60) was provided by Dr H. Hemmi (Tohoku University, Sendai). The human monoblast-like lymphoma cell line (U937) was provided by Dr K. Takeda (Showa University, Tokyo).

2.3. *Incubations of phagocytic cells with 25(OH)[^{3}H]D_3*

Phagocytic cells (7×10^6) were incubated with 1 μCi 25(OH)[^{3}H]D_3 in 3.5 ml of a serum-free medium for 1–36 h at 37°C under 5% CO_2-95% air. The serum-free medium consisted of a mixture of RPMI 1640, Dulbecco's modified Eagle's MEM and Ham's F-12 (Gibco, Grand Island, NY) (2:1:1) containing 2.219 mg/ml of sodium bicarbonate, 100 μg/ml of streptomycin sulfate, 100 U/ml of penicillin G potassium, 8.47 ng/ml of selenous acid, 110 μg/ml of sodium pyruvate and 1 μg/ml of human transferrin (Sigma, St. Louis, MO). After incubation, the cells and medium were extracted together [9], and the samples were applied to a preparative silica Sep-Pak cartridge column (Waters, Milford, MA) [10]. The fraction containing dihydroxy metabolites of vitamin D_3 was subjected to Waters HPLC, pump model 6000 A, equipped with a Finepak Sil column (0.46 × 25 cm, Jasco, Tokyo). The column was eluted with 10% 2-propanol in hexane at a flow rate of 1 ml/min. Fractions were collected every 30 s and the radioactivity of the eluate was measured with a liquid scintillation counter.

3. RESULTS

Alveolar macrophages incubated for 1 h with 25(OH)[^{3}H]D_3 produced 10-oxo-19-nor-25(OH)D_3 as a mixture of 5*E* and 5*Z* isomers (fig.1A). The natural 5*E* isomer was accompanied by the 5*Z* isomer photochemically produced, because a special precaution was not taken to avoid exposure

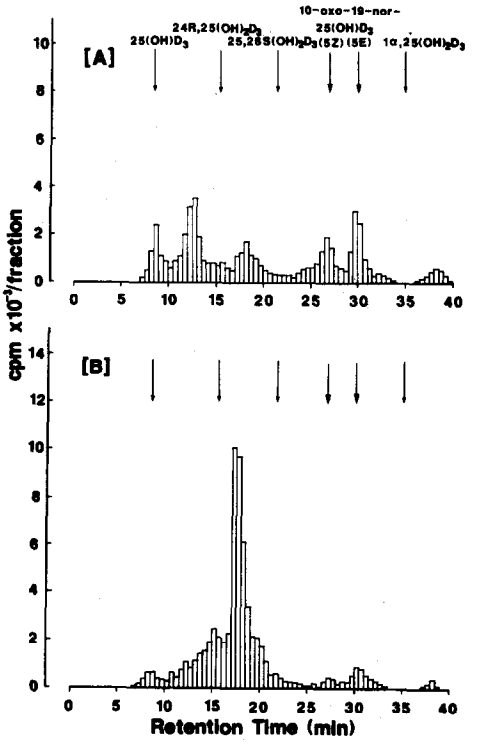

Fig.1. HPLC profiles of the metabolites of 25(OH)[^{3}H]D_3 produced by alveolar macrophages incubated with 25(OH)[^{3}H]D_3 for 1 h (A) and 24 h (B). Lipid extracts of the incubation mixture were first subjected to a silica Sep-Pak cartridge column. A radioactive fraction eluted with 60% ethyl acetate in *n*-hexane was applied to HPLC (Finepak Sil column, 0.46 × 25 cm, 10% 2-propanol in hexane, 1 ml/min, monitored at 265 nm). Arrows show the elution positions of the authentic vitamin D_3 compounds.

of the experimental vessels to room light during incubation and chromatographic separation. The production of the metabolite attained a maximum at 1 h and decreased thereafter (fig.2A). Formation of 10-oxo-19-nor-25(OH)D_3 was also observed when 25(OH)D_3 was allowed to stand for 1–72 h at 37°C under 5% CO_2-95% air in the medium without the cells (fig.2B). Formation of the metabolite in the medium alone increased time-dependently and attained a plateau at 48 h. The structures of (5*E*)- and (5*Z*)-10-oxo-19-nor-25(OH)D_3 were confirmed by ultraviolet (maximum at 310 nm), mass spectra [402(M^+), 384, 369, 359, 273 and 177], and chemical synthesis.

When alveolar macrophages were incubated with 25(OH)[^{3}H]D_3 for a longer time, another radioactive peak appeared at approx. 17 min (fig.1B). The production of the new metabolite increased time-dependently and reached a maximum at 12–24 h (fig.2A). This metabolite was not produced when 25(OH)D_3 was allowed to stand at 37°C in the medium without the cells.

The elution position of the new metabolite was

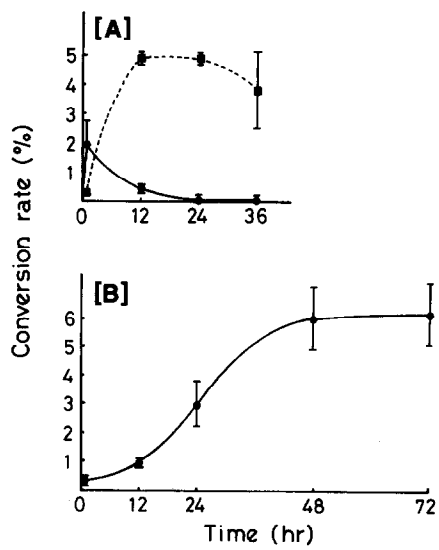

Fig.2. Time course of the change in the rate of conversion of 25(OH)D_3 to 10-oxo-19-nor-25(OH)D_3 (——) and the new metabolite (– – –). Alveolar macrophages were incubated with 25(OH)[^{3}H]D_3 for 1–36 h (A). 25(OH)[^{3}H]D_3 was allowed to stand in medium without macrophages for 1–72 h (B). Points and bars represent means ± SE of 3–6 independent sets of experiments.

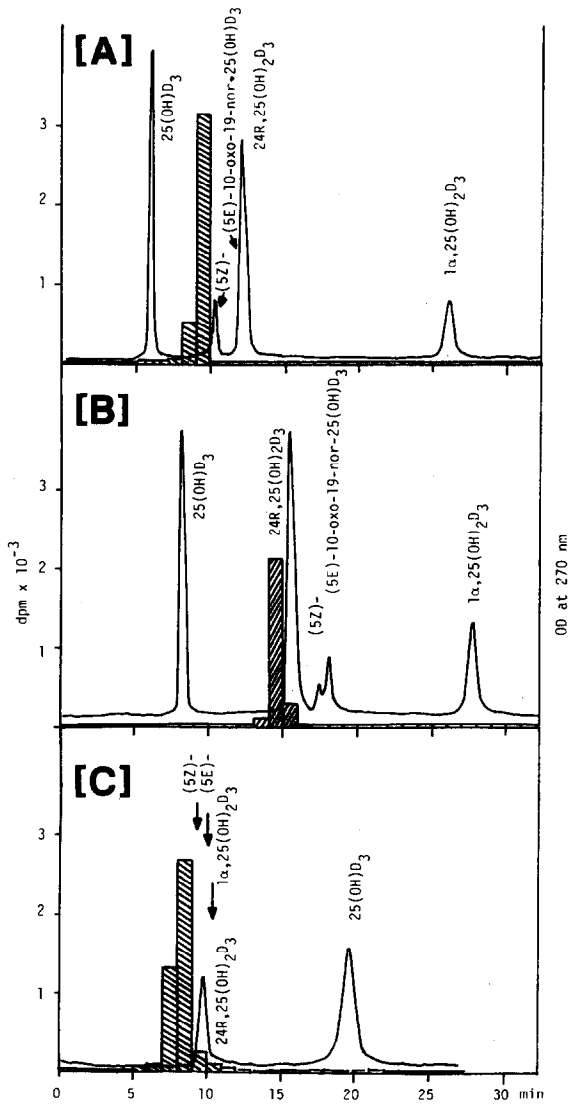

Fig.3. Cochromatography of the new metabolite with authentic vitamin D_3 compounds on three different HPLC systems. The radioactive peak eluting at 17 min on the HPLC system shown in fig.1B was pooled and cochromatographed with authentic vitamin D_3 compounds on three different HPLC systems: (i) Lichrosorb Si 60 column (0.46 × 25 cm), 2% methanol in dichloromethane, 1 ml/min (A); (ii) same column as in (i), hexane/dichloromethane/methanol (16:2:1), 1 ml/min (B); (iii) Finepak Sil C_{18} column (0.46 × 25 cm), 15% water in methanol, 1 ml/min (C). The solid line shows the absorbance at 270 nm and the bar represents radioactivity in each 1-min fraction. Arrows show the elution positions of the authentic compounds indicated.

compared with those of authentic vitamin D compounds in three different HPLC systems besides the one shown in fig.1: a straight-phase column with methanol-dichloromethane (fig.3A) and that with hexane-dichloromethane-methanol solvent systems (fig.3B), and a reverse-phase column with water-methanol (fig.3C). The new metabolite was eluted in the vicinity of 24*R*,25(OH)$_2$D$_3$ in these three HPLC systems, but did not comigrate to the same position of 24*R*,25(OH)$_2$D$_3$.

The time course of the production of the two metabolites (fig.2A) led us to examine the possibility that the new metabolite is derived from 10-oxo-19-nor-25(OH)D$_3$. 10-Oxo-19-nor-25(OH)-[^{3}H]D$_3$ was synthesized by allowing 25(OH)[^{3}H]D$_3$ to stand for 2 days at 37°C in the medium alone. When incubated with alveolar macrophages, 10-oxo-19-nor-25(OH)[^{3}H]D$_3$ was not converted to the new metabolite at any incubation times (not shown).

Metabolism of 25(OH)D$_3$ was also studied in several lines of mouse and human myeloid leukemia cells (M1, U937, and HL-60). All of these myeloid cells produced the two metabolites, 10-oxo-19-nor-25(OH)D$_3$ and the new metabolite. The mode of production of the two metabolites by these cells was similar to that by alveolar

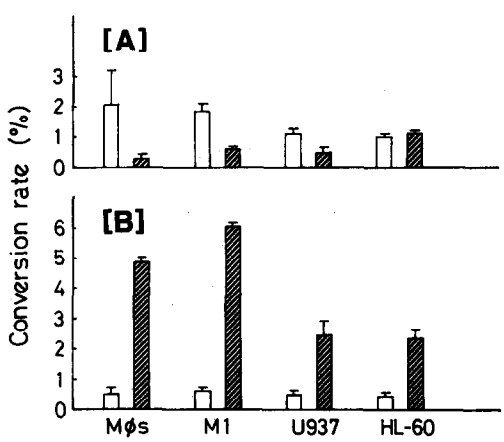

Fig.4. Conversion of 25(OH)D$_3$ to 10-oxo-19-nor-25(OH)D$_3$ (A) and the new metabolite (B). Phagocytic cells (7×10^6) (alveolar macrophages, M1, U937, and HL-60) were incubated with 1 μCi 25(OH)[^{3}H]D$_3$ for 1 h (empty columns) or 24 h (hatched columns). Columns and bars represent means ± SE of 3–4 independent sets of experiments.

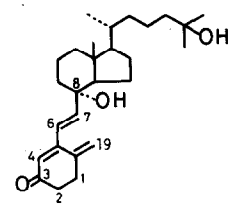

Fig.5. The structure of the new metabolite, 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one.

macrophages; 10-oxo-19-nor-25(OH)D$_3$ was the dominant metabolite at 1 h, while the new metabolite was the major metabolite at 24 h (fig.4).

The new metabolite was isolated by incubating 25(OH)D$_3$ with M1 cells and unequivocally identified as 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one (fig.5) on the basis of its ultraviolet (maximum at 295 nm and minimum at 245 nm), infrared (presence of a conjugated carbonyl group at 1660 cm^{-1}), mass (M$^+$: 414 *m/e*), and ^{1}H-NMR spectra. The stereochemistry of the hydroxyl group newly introduced into the 8-position was determined to be α by chemical synthesis. Details of the identification work have been submitted.

4. DISCUSSION

We found that the conversion of 25(OH)D$_3$ to 10-oxo-19-nor-25(OH)D$_3$ occurred in the presence and absence of phagocytic cells. The time course of the conversion of 25(OH)D$_3$ to 10-oxo-19-nor-25(OH)D$_3$ was, however, different between the presence and absence of alveolar macrophages. Formation of the metabolite attained a maximum at 1 h and decreased thereafter in the presence of macrophages, whereas it increased time-dependently in the absence of the cells. The rate of conversion of 25(OH)D$_3$ to the metabolite was much higher in the absence of macrophages. Using solubilized kidney mitochondria, Brown and DeLuca [11] have also reported that 10-oxo-19-nor-25(OH)D$_3$ is produced when 25(OH)D$_3$ is allowed to stand in a buffer alone and also in a reconstitution system without the P-450 fraction, but that its formation is suppressed by the addition of antioxidants. From these results they suggested that the production of 10-oxo-19-nor-25(OH)D$_3$ occurs via a mechanism involving peroxidation.

Our preliminary experiments have also indicated that the addition of divalent iron (Fe^{2+}) with oxygen gas causes the conversion of 25(OH)D_3 to (5*E*)-10-oxo-19-nor-25(OH)D_3 in the absence of phagocytic cells (unpublished). Phagocytic cells, however, somehow enhance this oxidation. These results confirm the suggestion of Brown and DeLuca [11].

Longer incubations (12–36 h) of phagocytic cells with 25(OH)[^{3}H]D_3 produced another metabolite eluting at 17 min on HPLC. The new metabolite was unequivocally identified as 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholesta-trien-3-one. In contrast to the production of 10-oxo-19-nor-25(OH)D_3, the new metabolite was formed only in the presence of phagocytic cells. In addition, the new metabolite was not produced when phagocytic cells were incubated with radioactive 10-oxo-19-nor-25(OH)D_3, suggesting that the new metabolite is formed from 25(OH)D_3 directly.

It is interesting that the new metabolite migrated in the vicinity of authentic 24*R*,25$(OH)_2D_3$ in the four different HPLC systems (figs 1,3). During the preparation of the present manuscript, Reichel et al. [12] reported that HL-60 cells exposed to 1α,25$(OH)_2D_3$ produced 24,25$(OH)_2D_3$ from 25(OH)D_3. In our study, phagocytic cells were not exposed to 1α,25$(OH)_2D_3$ before incubation with 25(OH)[^{3}H]D_3, and the production of 24,25-$(OH)_2$[^{3}H]D_3 was not observed. It is also interesting that Reichel et al. [12] did not find the new metabolite under their experimental conditions. Further studies are needed to define the relationship between the formation of 24,25$(OH)_2D_3$ and the new metabolite described here.

A recent report has indicated that 25(OH)D_3 is metabolized to 1α,25-$(OH)_2D_3$ in human alveolar macrophages treated in vitro with interferon-γ [13]. In this study, we isolated the two metabolites oxidized at the triene part, 10-oxo-19-nor-25(OH)D_3 and 8α,25-dihydroxy-9,10-seco-4,6,10(19)-cholestatrien-3-one. In the classical metabolism of vitamin D_3, the non-triene part was oxidized at the 1α, 24-, 25- and 26-positions. It is important to clarify the mechanism underlying the differences of these two types of oxidation. The biological significance of the new metabolite must also be elucidated in the future.

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